

Environmental arsenic exposure and sputum metalloproteinase concentrations

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Abstract

Exposure to arsenic in drinking water is associated with an increased rate of lung cancer. The objective of this study was to determine whether arsenic exposure at relatively low concentrations ($\sim 20 \mu\text{g/L}$) is associated with changes in biomarkers of lung inflammation, as measured by the ratio of sputum metalloproteinase and antiproteinase activity. A total of 73 subjects residing in Ajo and Tucson, Arizona were recruited for this cross-sectional study. Tap water and first morning void urine were analyzed for arsenic. Matrix metalloproteinase 2 (MMP-2), 9 (MMP-9) and tissue inhibitor of metalloproteinase 1 (TIMP-1) were measured in induced sputum. Household tap water arsenic levels in Ajo ($20.3 \pm 3.7 \mu\text{g/L}$) were higher than in those Tucson ($4.0 \pm 2.3 \mu\text{g/L}$), as were mean urinary total inorganic arsenic levels (29.1 ± 20.4 and $11.0 \pm 12.0 \mu\text{g/L}$, respectively). Log-normalized MMP-2, MMP-9, and TIMP-1 concentrations in sputum were not significantly different between towns. However, after adjusting for town, asthma, diabetes, urinary monomethylarsonic acid/inorganic arsenic, and smoking history, total urinary arsenic was negatively associated with MMP-2 and TIMP-1 levels in sputum and positively associated with the ratio of MMP-2/TIMP-1 and MMP-9/TIMP-1 in sputum. Increased sputum proteinase/antiproteinase activity suggests a potential toxic mechanism for low-level arsenic exposure.

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1. Introduction

Exposure to arsenic in drinking water has been associated with reduction in lung function (von Ehrenstein et al., 2005) and increased risk of lung, kidney, liver, and skin cancers (Chen et al., 1988, 1992). Although there have been extensive animal studies, the molecular mechanisms of low-concentration-arsenic-mediated toxicity are poorly understood. Several possible mechanisms including increased inflammation, oxidative stress, altered DNA methylation patterns, inhibition of DNA repair, and modulation of signal transduction pathways have been described (Liu et al., 2000; Yamanaka et al., 1990; Zhao et al., 1997).

As part of the lung inflammatory response, proteolytic enzymes including the matrix metalloproteinases (MMPs) are continually secreted in the airways. Two prominent metalloproteinases are matrix metalloproteinase 9 (MMP-9) and matrix metalloproteinase 2 (MMP-2). The activity of the metalloproteinases is regulated by metalloproteinase inhibitors, with tissue inhibitor of metalloproteinase 1 (TIMP-1) considered to be the major inhibitor (Okada et al., 1992; Murphy and Willenbrock, 1995). Both the absolute concentration of MMP-9 and its relative concentration, expressed as a ratio with TIMP-1, are considered sensitive indicators of increased inflammation in asthma (Vignola et al., 1998; Lim et al., 2000). Arsenic exposure has been associated with changes in MMP-9 in prostate cells (Achanzar et al., 2002) and keratinocytes (Cooper et al., 2004), and in unpublished studies of the lungs of mice exposed to arsenite in drinking water (Andrew et al., 2004).

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Ingested inorganic arsenic is methylated to arsenic species, such as monomethylarsonic acid (MMA) and dimethylarsenic acid (DMA), and increased methylation may exert influence on the carcinogenic potential of arsenic (Styblo et al., 2000). The ratio of DMA/MMA and the relative levels of MMA or DMA over inorganic arsenic are also considered indicators of biological methylation activity in humans (Chowdhury et al., 2002). The objectives of this study were to determine whether low-level ($\sim 20 \mu\text{g/L}$) arsenic exposure is associated with changes in biomarkers of lung inflammation, as measured by sputum MMP-2, -9, and TIMP-1 levels or their ratios, and whether these relationships are modulated by the extent of arsenic methylation.

2. Materials and methods

2.1. Study area and selection of households

This study was approved by the Institutional Review Board at the University of Arizona and was part of a larger study to evaluate the effects of a bottled water intervention on arsenic exposure through drinking water. Census blocks within census tracts were initially selected at random. Based on a probability proportional to size (PPS) sampling protocol (Lohr, 1999), our goal was to recruit no more than five households per census block. Our inclusion criteria required at least 3 years continuous residence in Ajo at the time of recruitment, use of tap water for drinking and food preparation, and no current smoking. In the town of Ajo we had difficulty recruiting a sufficient number of households using the PPS method, given our restrictive inclusion criteria, and we resorted to a census of the entire community. In Tucson, the five census tracts that most closely resembled the Ajo population in age distribution and percentage Hispanic residents in the 2000 census were selected. Two of the five census tracts were randomly selected and census blocks and households in these blocks were randomized. Again, we recruited up to five households per block. The majority of households in both communities that did not meet the inclusion criteria were disqualified due to their current use of bottled water. Household recruitment took place between June 2002 and August 2003.

2.2. Questionnaire

A baseline questionnaire based on the National Human Exposure Assessment Survey was administered by a trained interviewer in either English or Spanish at the time of sample collection. Information related to demographics, medical history, and occupational and environmental arsenic exposures was collected. Information on consumption of seafood and/or mushrooms during the prior 3-day period was collected to assess for other potential sources of arsenic exposure.

2.3. Sample collection

A first morning void urine sample was collected in two sterile 120-mL screw topped polypropylene containers, which were previously tested and determined to be arsenic free. Urine samples were processed within 2 h of collection. Urine pH was measured with pH indicator strips and samples were then transferred to sterile 50-mL conical vials. Samples were centrifuged at 2400 rpm for 20 min to separate cells from the supernatant, which was stored in 15-mL vials at -20°C before being transported to Tucson for storage at -80°C . Thawed urine supernatant was thoroughly mixed by vortex before being aliquoted for urinary arsenic and creatinine assay. Prior to actual analysis, samples were briefly centrifuged again to remove precipitates, providing a clear supernatant used for laboratory analyses.

Sputum induction was performed as previously described (Burgess et al., 2002). Subjects were asked to breathe a sterile 3% saline aerosol (Baxter, Deerfield, IL) generated using DeVilbiss Ultra-Neb 99HD

ultrasonic nebulizers (Somerset, PA) set on maximum output. Subjects were encouraged to cough up every 2 min for a period of 30 min and the sputum was collected in a 50-mL conical vial. Prior to each cough, subjects were asked to discard any saliva to minimize salivary contamination of sputum sample. Sputum samples were treated with 10% Sputolysin (Calbiochem, San Diego, CA) containing penicillin–streptomycin to inhibit bacterial growth. Supernatant was then separated by centrifugation at 1900 rpm for 15 min and stored at -20°C before being transferred to Tucson for storage at -70°C .

2.4. Arsenic and creatinine analysis

For each household, tap water from the cold water faucet in the kitchen was allowed to run for 1 min before collecting water in two sterile 50-mL conical vials. The samples were stored at 4°C until transported to the University of Arizona for arsenic analysis. An HPLC-ICP-MS speciation method was modified for the measurement of arsenic (Gong et al., 2001). The HPLC system consisted of an Agilent 1100 HPLC (Agilent Technologies, Inc.) with a reverse-phase C18 column (Prodigy 3 μ ODS (3), 150×4.60 mm; Phenomenex, Torrance, CA). The mobile phase (pH 5.85) contained 4.7 mM tetrabutylammonium hydroxide, 2 mM malonic acid, and 4% (v/v) methanol at a flow rate of 1.2 mL/min. The column temperature was maintained at 50°C . An Agilent 7500a ICPMS with a Babington nebulizer was used as the detector. The operating parameters were as follows: R_f power, 1500 W; plasma gas flow, 15 L/min; carrier flow, 1.2 L/min; and arsenic was measured at $75 m/z$. For total arsenic, an ASX500 auto sampler (CETAC Technologies, Omaha, NE) was used to introduce the samples into the Agilent 7500a ICPMS. The operating parameters were as follows: R_f power, 1500 W; plasma gas flow, 15 L/min; and carrier flow, 1.2 L/min. The acquisition parameters for arsenic were measured at m/z 75, terbium (IS) measured at m/z 159, points per peak were 3, dwell time for arsenic was 1.5 s, and the dwell time for Tb was 1.5 s.

Urinary total inorganic arsenic was defined as the sum of As^{3+} , As^{5+} , MMA, and DMA. Creatinine levels were estimated in urine samples using Jaffe's reaction method in a microplate format (Quidel Inc., San Diego, CA). Urinary arsenic concentration was adjusted for creatinine by expressing its level as a ratio with creatinine ($\mu\text{g/g}$ creatinine). In accordance with WHO recommendations (Mikheev, 1996), urine samples with creatinine concentrations below 30 mg/dL or above 300 mg/dL were excluded from the statistical analyses.

2.5. Arsenic and selenium analysis: toenails

As part of a cooperative project involving the University of Arizona and Dartmouth College, toenails were sent to Dartmouth College for analysis. Each toenail sample was washed with acetone, Triton X-100, and water to remove external contaminants. Afterward, the complete sample was weighed into a Teflon vial, 1 mL of concentrated HNO_3 was added, the vial was capped, and the sample was digested under pressure at 30°C in a microwave oven. The resulting solution was added to 4 mL of water and stored at 5°C until analysis. For arsenic analysis, this solution was diluted an additional four times with water and analyzed using an Agilent 7500c ICPMS instrument with an Octapole reaction cell pressurized with helium gas to remove potential interference from ArCl. The detection limit was $0.005 \mu\text{g/g}$. Similarly, for selenium analysis, the same procedure used for arsenic was adopted except in this case hydrogen was used as reaction gas and selenium was monitored using three isotopes $m/z = 76, 77$, and 78. The final result was the average of the results for all individual selenium isotopes. The detection limit for selenium was also $0.005 \mu\text{g/g}$.

2.6. Sputum analysis

All sputum supernatant samples were analyzed in duplicate for levels of total (active and zymogen form) MMP-2, MMP-9, and TIMP-1 using commercially available enzyme-linked immunosorbent assays (ELISA) (R&D Systems, Minneapolis, MN). A uniform initial sample dilution was

performed on all samples to maximize the number of samples with concentration within the standard range for MMP-9 and TIMP-1 ELISA. Three of the 73 samples (4.1%) were evaluated to have MMP-2 and TIMP-1 concentrations below the detectable limit, while only 1 sample (1.4%) was below detection limit when measuring for MMP-9. For statistical analysis, samples with metalloproteinase levels at less than detectable limits of ELISA were replaced with a value half the limit of detection for that assay. Total protein concentration was estimated using the biocinchoninic acid assay in a microplate (Sigma Aldrich, St. Louis, MO). Total urea in induced sputum was estimated using Urea Nitrogen reagent kit (Pointe Scientific Inc., Lincoln Park, MI).

2.7. Statistical analysis

SPSS 12.0 (Chicago, IL) and Stata 8.2 (College Station, TX) software were used for statistical analyses. For parametric tests, sputum metalloproteinase concentrations were normalized using natural log (ln) transformations. Independent-sample *t* tests were used to compare population characteristics by town and metalloproteinase concentrations by gender, race, town, diabetes (current vs. never), ever-asthma (yes vs. no), and past smoking (yes vs. no). Pearson's correlation analyses were used to assess correlations among the different sputum biomarkers.

Multiple linear regression models were used to assess the association of urinary arsenic and the various measures of sputum metalloproteinases, adjusting for potential confounders. For each of the outcome measures, variables found to be marginally significant in univariate regression analyses were included in a stepwise regression model using a backward elimination technique to decipher final models. Diagnostics were then performed for each model to assess whether assumption parameters for linear regression were met. To account for possible confounding effects, town, ever-asthma, and MMA/(As³⁺ + As⁵⁺) were included in each model, regardless of their statistical significance. Town was forced into the models to account for other environmental or biological differences not assessed in this study. Similarly, asthma was included to account and adjust for known lung inflammation associated with the disease, and the ratio of MMA/(As³⁺ + As⁵⁺) was used as a measure of active methylation of inorganic arsenic.

3. Results

3.1. Subjects

A total of 73 individuals, 40 from 33 households in Ajo and 33 from 30 households in Tucson, met inclusion criteria (Table 1). The study population ranged in age from 30 to 92 years and was predominantly female, over 50% non-Hispanic white, and college educated. The Tucson and Ajo populations did not differ significantly in age distribution, gender, race, asthma or diabetes diagnoses, or past smoking history. A substantial proportion of subjects in both towns reported ever having asthma and ever having diabetes. Seventy-two participants provided first morning void urine samples, and 56 (77%) provided induced sputum for analysis (33 from Ajo and 23 from Tucson). There were no significant differences in demographics, smoking history, or asthma or diabetes between subjects who did (*N* = 56) and did not (*N* = 17) provide sputum samples.

3.2. Water and urinary arsenic concentrations

Sixty-two tap water samples were collected. The mean concentration of arsenic in Ajo tap water was approxi-

Table 1
Descriptive statistics by town

	Ajo <i>N</i> (%)	Tucson <i>N</i> (%)
Number of subjects	40	33
Female	25 (63)	21 (64)
Age in years (mean ± SD)	61.8 ± 15.8	64.7 ± 14.5
Under 60 years	16 (40)	13 (39)
61–80 years	18 (45)	15 (46)
Over 80 years	6 (15)	5 (15)
Ethnicity		
Hispanic	16 (40)	10 (30)
White non-Hispanic	23 (57)	23 (70)
Other	1 (3)	0 (0)
Educational level		
Less than high school	7 (18)	4 (12)
High school graduate	13 (32)	9 (27)
College and beyond	20 (50)	20 (61)
Personal history		
Past smoking	18 (45)	12 (40)
Pre-existing illness		
Ulcer	2 (5)	2 (6)
Ever asthma	14 (35)	16 (48)
Ever diabetes	8 (20)	5 (15)

mately five times higher than that in Tucson. Tap water in Ajo is obtained entirely from a single ground water well. Tucson tap water is supplied by multiple wells. A comparison of mean urinary concentrations of various arsenic species by town is shown in Table 2. The concentrations of As³⁺, As⁵⁺, MMA, and DMA were summed to obtain the total urinary inorganic arsenic level, which was significantly higher in Ajo than in Tucson (*P* < 0.001). Due to a loss of 20 urine samples following arsenic analysis and prior to creatinine analysis, 1 subject who failed to provide urine, and the presence of creatinine levels > 300 or < 30 mg/dL in 9 samples, only 43 (69%) of the subjects had valid creatinine levels. However, creatinine-adjusted total inorganic arsenic showed the same trend by town (*P* < 0.001) as the unadjusted values. Both MMA and DMA species of urinary arsenic were significantly higher in Ajo than in Tucson. The ratios of MMA/(As³⁺ + As⁵⁺) and DMA/(As³⁺ + As⁵⁺) were higher in Tucson than in Ajo, although these differences were not statistically significant. No difference in the ratio of urinary DMA/MMA by town was found.

3.3. Induced sputum

Mean concentrations of sputum metalloproteinases in Ajo and Tucson are also presented in Table 2. Natural-log-normalized sputum concentrations of MMP-2, MMP-9, and TIMP-1 were significantly positively correlated (*P* < 0.001). The mean sputum urea concentration was approximately twice as high in the Ajo population as in the Tucson population (*P* < 0.023), but there was no difference in

Table 2
Concentration of arsenic species in water and urine and sputum metalloproteinase levels by town

Variable	Ajo (mean \pm SD)	Tucson (mean \pm SD)	Sig. <i>P</i> value ^a
Water (<i>n</i>)	33	29	
Total water As ($\mu\text{g/L}$)	20.3 \pm 3.7	4.0 \pm 2.3	<0.001
Urine (<i>n</i>)	40	32	
Total urinary As ($\mu\text{g/L}$) ^b	28.3 \pm 18.6	14.1 \pm 13.5	<0.001
Total urinary inorganic As ($\mu\text{g/L}$) ^c	29.1 \pm 20.4	11.0 \pm 12.0	<0.001
Urinary As ³⁺ + As ⁵⁺ ($\mu\text{g/L}$)	8.8 \pm 9.6	4.5 \pm 8.7	0.053
Total urinary MMA ($\mu\text{g/L}$)	2.9 \pm 3.7	1.0 \pm 1.1	<0.001 ^d
Total urinary DMA ($\mu\text{g/L}$)	17.5 \pm 12.5	5.5 \pm 4.4	<0.001 ^d
Urinary DMA/MMA	13.1 \pm 29.0	14.4 \pm 23.1	0.998 ^d
Urinary MMA/(As ³⁺ + As ⁵⁺)	0.70 \pm 0.60	1.89 \pm 3.20	0.383 ^d
Urinary DMA/(As ³⁺ + As ⁵⁺)	5.1 \pm 5.9	10.8 \pm 14.1	0.295 ^d
Creatinine-adjusted urinary As ($\mu\text{g/g}$) ^c	30.6 \pm 13.2	13.7 \pm 9.6	<0.001
Sputum (<i>n</i>)	33	23	
Sputum MMP-2 (ng/mL)	2.9 \pm 4.4	4.3 \pm 5.1	0.155 ^d
Sputum MMP-9 ($\mu\text{g/mL}$)	0.7 \pm 1.0	0.7 \pm 1.3	0.701 ^d
Sputum TIMP-1 (ng/mL)	199 \pm 208	202 \pm 244	0.842 ^d
Sputum protein (mg/mL)	8.0 \pm 3.7	8.9 \pm 5.3	0.443 ^d
Sputum urea (mg/mL)	64.2 \pm 92.5	35.7 \pm 12.8	0.023 ^d
Sputum MMP-2/TIMP-1	0.06 \pm 0.09	0.07 \pm 0.09	0.303 ^d
Sputum MMP-9/TIMP-1	13.9 \pm 24.5	19.4 \pm 38.7	0.986 ^d
Toenail (<i>n</i>)	38	33	
Arsenic ($\mu\text{g/g}$)	0.51 \pm 0.72	0.17 \pm 0.21	0.001 ^d
Selenium ($\mu\text{g/g}$)	1.42 \pm 1.78	1.29 \pm 0.71	0.125

^aCompared using independent-sample *t* test with normally distributed test variable, unless specified otherwise.

^bTotal urinary arsenic was measured using Agilent 7500a ICPMS.

^cTotal urinary inorganic arsenic = (As³⁺ + As⁵⁺ + MMA + DMA). Individual species of arsenic were measured using the HPLC-ICP MS method.

^dThe comparisons were performed using two-sample *t* test with log-normalized variable data.

^eValid values *N* = 14 for Ajo and *N* = 29 for Tucson.

sputum protein concentrations in the two populations. There were also no differences in log-transformed MMP-2, MMP-9, TIMP-1, MMP-2/TIMP-1, and MMP-9/TIMP-1 by town, gender, race, or ever-smoking status. Diabetics had significantly higher mean levels of log MMP-2 (1.49 \pm 1.12 ng/mL) than nondiabetics (0.40 \pm 1.20 ng/mL) (*P* = 0.014). Similarly, diabetics had higher levels of log MMP-9 (6.59 \pm 0.97 $\mu\text{g/mL}$) than nondiabetics (5.67 \pm 1.30 $\mu\text{g/mL}$) (*P* = 0.048). Concentrations of log TIMP-1 were not significantly different in diabetics (5.34 \pm 0.90 ng/mL) and nondiabetics (3.93 \pm 2.08 ng/mL) (*P* = 0.052). For analyses stratified by town, Tucson individuals who reported ever having asthma had a significantly lower mean log-transformed MMP-9/TIMP-1 ratio (*P* < 0.001). Higher mean concentrations of sputum TIMP-1 and a lower mean ratio of MMP-2/TIMP-1 were also observed in ever-asthmatics who resided in Tucson but not in Ajo. Similar statistical significance levels were seen with protein-adjusted and urea-adjusted metalloproteinase levels.

3.4. Relation of sputum metalloproteinases and urinary arsenic

Total urinary inorganic arsenic was positively correlated with log MMP-9/TIMP-1 ratio in sputum (Pearson's

ρ = 0.351, *P* = 0.009) (Fig. 1) and negatively correlated with the log of sputum levels of TIMP-1 (Pearson's ρ = -0.269, *P* = 0.050) (Fig. 2). These correlations were not significant using creatinine-adjusted arsenic concentrations, perhaps due to the small number of subjects with available creatinine measurements. The urinary DMA/MMA ratio was positively associated with log values of sputum MMP-2, and negatively associated with log MMP-9, and it had a marginally significant positive association with TIMP-1, indicating both pro- and anti-inflammatory effects of increased secondary methylation.

Multiple linear regression models were utilized to assess the effects of arsenic exposure on sputum concentrations of MMP-2, MMP-9, MMP-2/TIMP-1, and MMP-9/TIMP-1, after adjusting for potential confounders (Table 3). Total urinary inorganic arsenic was a significant predictor of decreased levels of sputum MMP-2 and TIMP-1 and increased ratios of MMP-2/TIMP-1 and MMP-9/TIMP-1. The ratio of MMA over the sum of As³⁺ and As⁵⁺ was negatively associated with sputum MMP-2, MMP-9, and TIMP-1. When evaluated in the regression models, replacing MMA/(As³⁺ + As⁵⁺) with either DMA/MMA or DMA/(As³⁺ + As⁵⁺) did not reveal any significant associations with sputum metalloproteinase levels. When creatinine-adjusted total inorganic urinary arsenic levels were used in

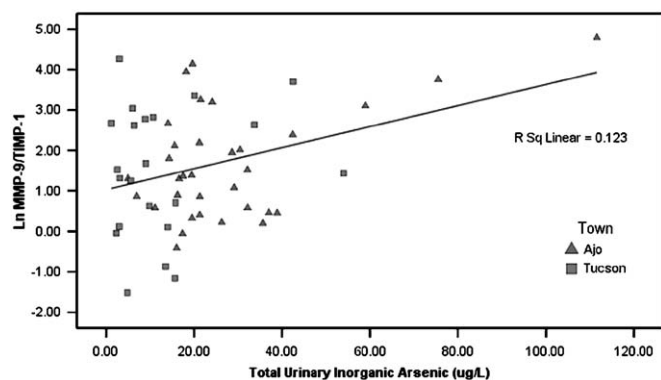


Fig. 1. Total urinary inorganic arsenic and ln MMP-9/TIMP-1.

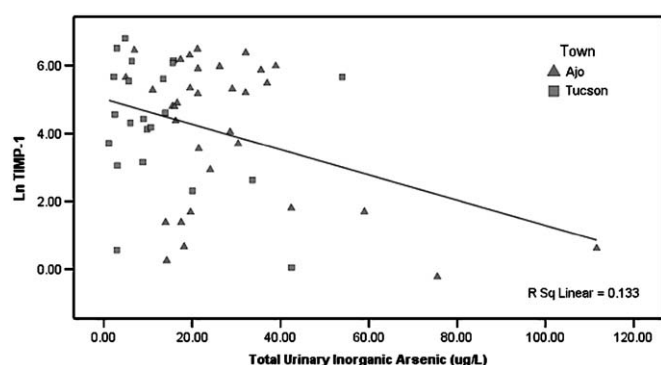


Fig. 2. Total urinary inorganic arsenic and ln TIMP-1.

these models, only the positive relationship between diabetes and MMP-2 and TIMP-1 remained significant.

Statistical models excluding the three subjects whose sputum values were below the detectable limit were run. For all variables in each of the five models, β coefficients were similar in direction and magnitude to those values reported in Table 3; however, two variables showed a change in statistical significance. The relationship between diabetics and TIMP-1 became nonsignificant ($P = 0.062$), whereas the association between town and MMP-2/TIMP-1 showed statistical significance ($P = 0.041$).

Univariate analysis of log-transformed toenail arsenic with log MMP-2 indicated a statistically significant negative correlation; however, the significance was lost when adjusted for potential confounders in the multiple regression models. All other associations between toenail arsenic and sputum metalloproteinase concentrations were nonsignificant. In addition, selenium levels in toenail were not associated with sputum metalloproteinase concentrations in the univariate comparisons or in the regression models.

4. Discussion

This is the first published study to find a measurable effect of low-level arsenic exposure on sputum metalloproteinase concentrations in humans. MMPs and tissue inhibitor of metalloproteinase are sensitive markers of lung inflammation in humans. In our study, urine concentration of total inorganic arsenic was a significant

Table 3
Regression models

Model	N	Adjusted R^2	Outcome variable	Predictor variables	β -Coefficient	P value
1	53	0.335	ln MMP-2	Town	0.588	0.065
				Asthma	0.122	0.694
				History of smoking	0.631	0.027
				Diabetic	1.370	0.001
				Total urinary inorganic As MMA/(As ³⁺ + As ⁵⁺)	−0.018	0.022
2	55	0.059	ln MMP-9	MMA/(As ³⁺ + As ⁵⁺)	−0.137	0.021
				Town	0.135	0.727
				Asthma	−0.271	0.446
3	55	0.158	ln TIMP-1	Total urinary inorganic As MMA/(As ³⁺ + As ⁵⁺)	−0.017	0.082
				MMA/(As ³⁺ + As ⁵⁺)	−0.167	0.025
				Town	0.010	0.985
				Asthma	0.364	0.485
				Diabetic	1.678	0.018
4	55	0.136	ln (MMP-2/TIMP-1)	Total urinary inorganic As MMA/(As ³⁺ + As ⁵⁺)	−0.049	0.001
				MMA/(As ³⁺ + As ⁵⁺)	−0.259	0.011
				Town	0.618	0.092
				Asthma	−0.085	0.798
5	55	0.106	ln (MMP-9/TIMP-1)	Total urinary inorganic As MMA/(As ³⁺ + As ⁵⁺)	0.028	0.004
				MMA/(As ³⁺ + As ⁵⁺)	0.120	0.083
				Town	0.178	0.673
				Asthma	−0.151	0.696
				Total urinary inorganic As MMA/(As ³⁺ + As ⁵⁺)	0.031	0.005
				MMA/(As ³⁺ + As ⁵⁺)	0.119	0.136

predictor of protease/antiprotease concentrations in the lungs, measured by the ratios of MMP-2 over TIMP-1 and MMP-9 over TIMP-1. These relationships may be largely attributable to the inverse relation between urinary inorganic arsenic and sputum TIMP-1.

No other human studies of arsenic exposure and lung inflammatory markers could be identified. In unpublished experiments on mice, long-term (8 weeks) exposure to 10 or 50 ppb of arsenic in drinking water led to increased mRNA expression of MMP-9 and decreased expression of MMP-2 in the lungs, while TIMP-1 mRNA levels were unchanged (Andrew et al., 2004). In other tissues, increased secretion of MMP-9 following arsenic exposure was reported in cell cultures of prostate cells (Achanzar et al., 2002) and keratinocytes (Cooper et al., 2004). In humans, arsenic exposure has been associated with a decrement in FEV₁ associated with long-term exposure at drinking water concentrations of less than 500 µg/L (von Ehrenstein et al., 2005). Increased inflammation is one potential explanation for this effect although other mechanisms may also be at work.

The Tucson and Ajo populations were exposed, respectively, to mean levels of arsenic in drinking water at ~4 and ~20 µg/L. Although there were significant differences between Tucson and Ajo in most measures of urinary arsenic, we found no differences in mean sputum metalloproteinase concentrations by town. Differences in quantities of tap water ingested through drinking and cooking, characterized by the wide range in urinary arsenic concentration among individuals within, may have contributed to our inability to find significant differences in inflammatory biomarkers by town.

Predominant sources of MMP-2 in the lung include macrophages and endothelial cells (Frisch and Morisaki, 1990). Predominant sources of MMP-9 include neutrophils and eosinophils (Devarajan et al., 1992; Schwingshackl et al., 1990) although MMP-9 can also be induced in several other cell types including endothelial cells, fibroblasts, and alveolar cells (Nagese and Woessner, 1999). Activity of MMP-2 and MMP-9 is inhibited by several intrinsic mediators including TIMP-1 to -4 (Butler et al., 1999; O'Connell et al., 1994; Stetler-Stevenson et al., 1989; Stratmann et al., 2001). Increasing evidence suggests a key role for at least some of the MMPs in the development of chronic and acute lung disease (Finlay et al., 1997; Ohnishi et al., 1998; Tanaka et al., 2000). MMPs play a crucial role in the trafficking of inflammatory cells in vitro (Leppert et al., 1995; Declaux et al., 1996) and may be involved in inflammatory cell migration in vivo (Kumagai et al., 1999). Previous studies have reported in vitro changes in MMP-9/TIMP-1 ratios as an effect of the anti-inflammatory cytokine interleukin-10 (Lim et al., 2000; Lacraz et al., 1995). Hence, the ratio of MMP-9 to TIMP-1, rather than absolute MMP-9 levels, may provide a better representation of the relative inflammatory status of airways. In our study, MMP-9/TIMP-1 was significantly associated with increased urinary arsenic concentration, while MMP-9 was not. A similar argument can be used to support the use of

MMP-2/TIMP-1 in evaluation of inflammation, rather than the MMP-2 alone. This is particularly important as our study found a negative association between urinary arsenic and MMP-2 but a positive association between urinary arsenic and MMP-2/TIMP-1 after adjusting for potential confounders. Unlike urinary inorganic arsenic, toenail arsenic levels were not associated with the ratio of protein/antiprotease concentrations. Toenail arsenic levels give a measurement of arsenic exposure occurring months previously while urinary arsenic levels provide a measure of very recent exposure. If sputum metalloproteinase concentrations reflect more recent exposure, then urinary arsenic would be a more appropriate biomarker.

The effects of arsenic at low doses are not well understood. Many studies have failed to show a dose-response relationship between incidence rates of various cancers and exposure to low levels (~10 µg/L) of inorganic arsenic (Moore et al., 2002; Bates et al., 1995; Steinmaus et al., 2003; Lamm et al., 2004; Karagas et al., 2001), making it difficult to quantify the lowest carcinogenic dose of inorganic arsenic. Several molecular mechanisms have been hypothesized to describe the biological effects of arsenic including oxidative damage, alterations in DNA methylation or repair enzymes, chromosomal damage, and changes in signal transduction pathways. Our study demonstrated dose-dependent changes in inflammatory markers at arsenic exposure concentrations at and below 20 µg/L.

Arsenic methylation, which was until recently considered a detoxification mechanism, is speculated to be associated with amplification of arsenic-mediated toxicity (Yamamoto et al., 1995, 1997; Wanibuchi et al., 1996; Gebel, 2002). All arsenic species have been shown to possess cytotoxic effects, with trivalent inorganic arsenic being the most and pentavalent DMA the least toxic (Styblo et al., 2000). The extent of methylation, indicated in this study by the MMA/(As³⁺ + As⁵⁺) ratio, may alter the arsenic-mediated toxicity potential at a given exposure level and may affect susceptibility of individuals to adverse health effects of arsenic exposure.

The relation between diabetes and sputum MMPs in our study is complex. Without adjusting for other variables, sputum concentrations of MMP-2 and MMP-9 were increased in diabetics. After adjusting for potential confounders in multiple regression models, MMP-2 and TIMP-1 were found to be positively associated with diabetes. No previous studies have measured sputum or bronchoalveolar lavage fluid concentrations of these mediators in diabetics. However, plasma MMP-9 in type-II diabetics was elevated in comparison with normal volunteers, while plasma MMP-2 was either higher or unchanged (Signorelli et al., 2005; Maxwell et al., 2001). In a separate study the levels of urinary MMP-9 in patients with diabetic nephropathy increased in accordance with the clinical stage of the disease (Tashiro et al., 2004). In contrast to our study evaluating sputum, plasma TIMP-1 concentrations were significantly elevated in diabetic patients compared to controls and serum levels of TIMP

in diabetic patients with microalbuminuria and proteinuria were significantly higher than those in patients without excessive urine protein (Maxwell et al., 2001; Kanauchi et al., 1996).

There were a number of limitations to our study. The mean age of study participants was over 60 years. While the exact association between age and inflammation is unknown, the current study results may be generalizable to a younger population. There were only a limited number of urine samples available for creatinine analysis, although creatinine adjustment is not necessarily preferred (Hinwood et al., 2002). A well-documented source of exposure to arsenic is through diet, especially fish (Moschandreas et al., 2002) and consumption of foods grown in arsenic-contaminated soils (EPA website, 2005), and these exposures were not directly measured in our study. However, using measurements of total inorganic arsenic rather than the total arsenic concentrations should have reduced confounding effects of seafood ingestion. Although not addressing food intake, a previous study in two Arizona mining towns did not demonstrate an association between arsenic in house dusts with urinary arsenic concentrations in adults (Hysong et al., 2003). Although the MMP-2/TIMP1 ratio has been used as a predictor of inflammatory status of liver and as an indicator of response to interferon therapy in HCV infection (Kasahara et al., 1997; Lichtinghagen et al., 2000), other TIMPs, most notably TIMP-2, a major inhibitor of MMP-2 activity, were not measured (Murphy and Willenbrock, 1995). Activity and differential concentrations of active and inactive components of metalloproteinases were not measured due to the need to store samples following field collection prior to transport to a central laboratory facility. Although we were able to measure sputum MMP and TIMP concentrations, sputum cell count and differential data were not available.

In conclusion, urinary arsenic was positively correlated with increased sputum proteinase/antiproteinase protein levels, apparently through reductions in TIMP-1. The extent of arsenic methylation, defined in this study by the ratio $\text{MMA}/(\text{As}^{3+} + \text{As}^{5+})$, also predicted the concentration of metalloproteinases in some models. Therefore, we suggest that chronic exposure to arsenic through drinking water and the extent of arsenic methylation, measured as total urinary inorganic arsenic and urinary $\text{MMA}/(\text{As}^{3+} + \text{As}^{5+})$, respectively, may be important predictors of lung metalloproteinase concentrations. Additional studies are necessary to confirm these results in other arsenic-exposed populations and at additional exposure levels.

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